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Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway

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The stress-inducible protein heme oxygenase-1 provides protection against oxidative stress. The anti-inflammatory properties of heme oxygenase-1 may serve as a basis for this cytoprotection. We demonstrate here that carbon monoxide, a by-product of heme catabolism by heme oxygenase, mediates potent anti-inflammatory effects. Both *in vivo* and *in vitro*, carbon monoxide at low concentrations differentially and selectively inhibited the expression of lipopolysaccharide-induced pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β, and macrophage inflammatory protein-1β and increased the lipopolysaccharide-induced expression of the anti-inflammatory cytokine interleukin-10. Carbon monoxide mediated these anti-inflammatory effects not through a guanylyl cyclase–cGMP or nitric oxide pathway, but instead through a pathway involving the mitogen-activated protein kinases. These data indicate the possibility that carbon monoxide may have an important protective function in inflammatory disease states and thus has potential therapeutic uses.

Heme oxygenase (HO) is the rate-limiting enzyme in the oxidative degradation of heme into bilirubin[1]. Whereas HO-2 and HO-3 are constitutively expressed, HO-1 is the inducible isoform that provides protection against oxidative stress[2]. Recent analyses of HO-1-null mice as well as the first reported HO-1-deficient human have strengthened the emerging paradigm that HO-1 is indeed an important molecule in the host's defense against oxidant stress, and have also emphasized the potent anti-inflammatory properties of HO-1: Both mice and humans deficient in HO-1 expression have a phenotype of an increased inflammatory state[3,4]. The anti-inflammatory effects of HO-1 in those studies further confirmed published data, albeit from indirect studies, that HO-1 imparts potent anti-inflammatory effects[5,6]. Furthermore, exogenous administration of HO-1 by gene transfer into rat lung not only protects the rats from a lethal concentration of oxygen but also mediates potent anti-inflammatory effects in the lung[7].

Septic shock and sepsis syndrome, resulting from excessive stimulation of immune cells, particularly monocytes and macrophages, remains one of the leading causes of death in hospitalized patients[8]. The pathophysiological changes seen in sepsis are often not due to the infectious organism itself but instead to the uncontrolled production of pro-inflammatory cytokines and chemokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and macrophage inflammatory protein (MIP)-1β. Accumulation of these mediators leads to leukocyte recruitment, capillary leak and tissue damage, ultimately producing the lethality of sepsis[9,10]. Lipopolysaccharide (LPS), a constituent of the Gram-negative bacterial cell wall, is the leading cause of sepsis and when administered experimentally to macrophages or mice mimics the same inflammatory responses. After LPS administration, there is a rapid but transient increase in these pro-inflammatory mediators, which are subsequently down-modulated by a battery of anti-inflammatory cytokines, including IL-10 and IL-1, which inhibit the synthesis of the pro-inflammatory cytokines and chemokines[11]. LPS initially binds to the CD14 and toll-like receptor 2 (or 4) at the cell surface[12,13] and activates the mitogen-activated protein (MAP) kinase pathways, including p38, p42/p44 extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK)[14–16]. The relationship between the activation of these signaling molecules, downstream cytokine expression, and physiologic function represents an active line of investigation.

Using this well-established model of LPS-induced inflammation, we determined whether carbon monoxide (CO), one of three main byproducts of the catabolism of heme by HO-1, has potent anti-inflammatory effects and mediates much of the anti-inflammatory effects seen with HO-1. We demonstrate here that CO acts as a potent anti-inflammatory molecule in

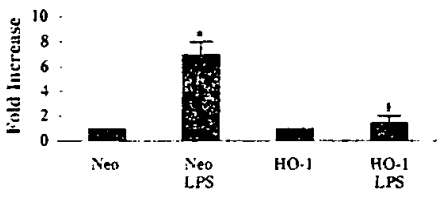

Fig. 1 Effects of overexpression of HO-1 in macrophages on LPS-induced TNF-α production. RAW cells transfected with neo (empty vector) or overexpressing HO-1 were treated with 1 ng/ml LPS, and TNF-α was measured in collected media by ELISA. *, $P < 0.0001$, compared with neo alone; § $P < 0.005$, compared with neo and LPS.

in vitro and *in vivo*. CO selectively inhibits the expression of the pro-inflammatory cytokines TNF-α, IL-1β and MIP-1β and increases production of the anti-inflammatory cytokine IL-10. We also show here that CO mediates these anti-inflammatory effects independent of nitric oxide[17] or cGMP, which is generally believed to mediate the effects of CO in other well-established vascular and neuronal cell culture systems[18,19]. Instead, our data indicate that CO mediates these anti-inflammatory effects specifically through the MAP kinases, in particular the MAP kinase kinase (MKK) 3/p38 pathway.

Overexpression of HO-1 inhibits TNF-α production

We generated macrophage cell lines (RAW 264.7) overexpressing HO-1 using a plasmid with HO-1 expression driven by pSFFV–long terminal repeat. We isolated nine clones and checked their HO-1 expression by western blot analysis. They had more HO-1 expression than did control cells transfected with the neomycin gene (data not shown). We exposed cells to 1 ng/ml LPS and measured by enzyme-linked immunosorbent assay (ELISA) the amount of TNF-α produced. As expected, there was substantial induction of TNF-α production in the control cells. In contrast, cells overexpressing HO-1 produced significantly less TNF-α after LPS treatment than did control cells (Fig. 1). Identical experiments in a second RAW 264.7 cell line clone overexpressing HO-1 showed similar results (data not shown).

CO targets pro- and anti-inflammatory cytokines *in vitro*

After showing that macrophages overexpressing HO-1 had reduced LPS-induced production of TNF-α (Fig. 1), we determined whether exposure of cells to a low concentration of CO could elicit similar responses in these same macrophages. We exposed wild-type RAW 264.7 macrophages to 1 μg/ml LPS in the presence or absence of 250 parts per million (p.p.m.) CO, and assessed TNF-α production by ELISA. As expected, cells exposed to LPS alone had more TNF-α. However, cells exposed to LPS in the presence of CO showed significantly less TNF-α (Fig. 2*a*), and this was concentration-dependent (range, 10–500 p.p.m.; data not shown). CO also produced similar inhibitory effects on the inflammatory cytokines IL-1β and MIP-1β (Fig. 2*b* and *c*), whereas it did not affect the chemokines MIP-2 (Fig. 2*d*), JE (the human homolog of monocyte chemotactic protein-1), gamma interferon or KC (melanoma growth-stimulating protein)(data not shown). In terms of the balance of pro- and anti-inflammatory cytokines, 250 p.p.m. CO significantly increased the LPS-induced accumulation of the anti-inflammatory cytokine IL-10 (Fig. 2*e*).

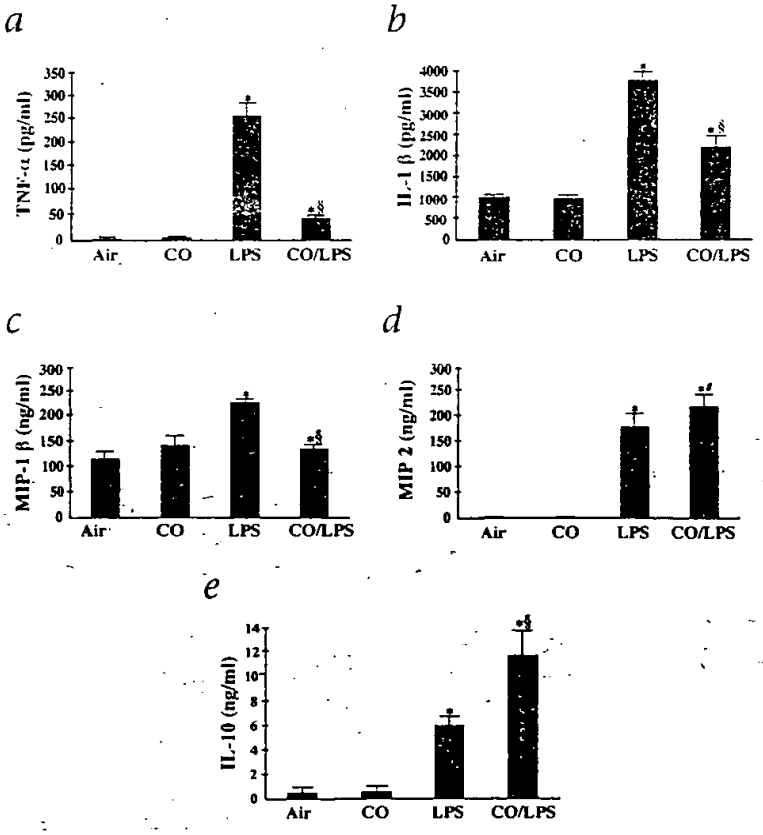

Fig. 2 Effects of CO on LPS-induced cytokine production *in vitro*. RAW 264.7 macrophages were pretreated with 250 p.p.m. CO before being treated with LPS. Culture media was collected and analyzed by ELISA. *a*, TNF-α. §, $P < 0.0001$, compared with LPS. *b*, IL-1β. §, $P < 0.001$, compared with LPS. *c*, MIP-1β. §, $P < 0.005$, compared with LPS. *d*, MIP-2. #, $P < 0.3$, compared with LPS. *e*, IL-10. §, $P < 0.05$ compared with LPS. *, $P < 0.001$, compared with air or CO alone (*a*–*e*). Data represent the mean value ± s.e.m. of six independent experiments.

CO targets pro- and anti-inflammatory cytokines *in vivo*

To assess whether the different effects of CO on pro-inflammatory and anti-inflammatory cytokines seen *in vitro* also occurred *in vivo*, we administered a sublethal dose of LPS (1 mg/kg) to mice in the presence or absence of CO (using the same concentrations used *in vitro*). Mice also produced significantly less TNF-α in the presence of CO, and CO attenuated LPS-induced production of TNF-α *in vivo* in a concentration-dependent manner (Fig. 3*a*), with a median effective concentration of 69.9 p.p.m. Consistent with our *in vitro* results, serum IL-10 increased in response to LPS in the presence of 250 p.p.m. CO *in vivo* (Fig. 3*b*). To determine whether hypoxia was involved in the inhibitory effects of CO on LPS-induced TNF-α production, we administered 1 mg/kg LPS to a separate group of mice in the absence or presence of hypoxic conditions (10% oxygen). There were no substantial effects on LPS-induced TNF-α production (3,800 ± 800 pg/ml, air control; 4,200 ± 500 pg/ml, hypoxia).

CO exerts effects by a cGMP-independent pathway

To delineate the possible mechanism by which CO exerts its effects, we determined whether the response to CO involved the

Table 1 Effects of CO on cGMP and LPS-induced TNF-α accumulation in RAW 264.7 cells

Treatment	cGMP (pmol/mg protein)	TNFα (pg/ml)
Air	4.25 ± 0.5	12 ± 10
CO	3.95 ± 0.6	15 ± 5
LPS	ND	660 ± 60*
CO/LPS	ND	230 ± 40**
8-Br-cGMP	ND	18 ± 6
8-Br-cGMP/LPS	ND	530 ± 35
VSM/Air	0.5 ± 0.4	ND
VSM/CO	8.1 ± 2.0***	ND

*, $P < 0.001$ compared with CO, Air, 8-Br-cGMP; **, $P < 0.03$ compared with LPS; ***, $P < 0.001$ compared with air. VSM, vascular smooth muscle cells; ND, not done.

guanylyl cyclase–cGMP pathway, which mediates the biological effects of CO in vascular and neuronal cells[18,19]. We exposed macrophages and vascular smooth muscle cells (used as a positive control) to 250 p.p.m. CO. After 2 hours of exposure, we analyzed cell lysates for cGMP levels by radioimmunoassay. Smooth muscle cells showed a 1,600% increase in cGMP content after exposure to CO ($P < 0.001$), whereas RAW 264.7 macrophages showed no significant increase in cGMP after exposure to CO (Table 1). To confirm the lack of involvement of cGMP in CO-treated macrophages, we added 1mM 8-Br-cGMP, an analog of cGMP that is non-degradable by phosphodiesterase yet maintains similar functional activity, to the macrophage culture followed by LPS to observe its effects on production of TNF-α. Macrophages exposed to CO showed no increase in cGMP levels, whereas 8-Br-cGMP had no effect on LPS-induced production of TNF-α (Table 1).

CO exerts effects by a NO-independent pathway

Given our observation that CO exerts anti-inflammatory effects through a cGMP-independent pathway, and knowing that CO can bind the heme moiety of nitric oxide synthase (NOS) and thereby modulate nitric oxide (NO) production[20], we investigated whether NO could indirectly mediate the anti-inflammatory effects seen with CO. We pretreated RAW 264.7 macrophages with L-NAME (N-nitro-L-arginine methyl ester; 10 μM), a selective inhibitor of NOS, before exposing cells to 1 μg/ml LPS in the presence or absence of CO. Cells pretreated with L-NAME in the presence of CO and LPS showed an inhibition of TNF-α production similar to that of cells exposed to CO and LPS only (74% and 78% inhibition, respectively). Further studies of LPS-induced NO production in RAW 264.7 cells showed no increases in either nitrate or nitrite levels at 1 hour after LPS administration in either the presence or absence of CO. Nitrite and nitrate levels did increase considerably (5,000%), but only after 16 hours following administration of LPS, compared with that in untreated control cells. This data further support the idea that CO could not be acting indirectly through NO to modulate production of TNF-α.

Modulation of MAP kinases by CO

Given that CO acts through a cGMP- and NO-independent pathway, we sought a possible alternative mechanism by which CO might mediate anti-inflammatory actions. Administration of LPS to macrophages results in the activation of the MAP kinase pathways[13–16]. We first confirmed activation of the p38, ERK1/ERK2 and JNK pathways in macrophages treated with LPS (Fig. 4, lane 2). In the presence of 250 p.p.m. CO, LPS-induced activation of the MAP kinases ERK1/ERK2 and JNK was not affected; however, p38 MAP kinase activation was significantly increased (Fig. 4*c*, lane 4). LPS also increased MKK3 and MKK6, kinases upstream of p38, in the presence of CO in RAW 264.7 cells, compared with their levels in cells treated with LPS alone (data not shown).

CO mediates effects by the MKK3 MAP kinase pathway *in vivo*

As the p38 MAP kinase pathway was activated by LPS and increased by CO, we tested whether attenuation of this pathway led to a loss of the response. There are three MKKs that activate the p38 MAP kinase: MKK3, MKK4 and MKK6 (refs. 21,22). Because macrophages deficient in MKK3 would have deficient p38 activation in response to LPS, and given that MKK3 mediates the activation of p38, we administered LPS to *Mkk3*$^{-/-}$ mice (Genome DataBase designation, *Map2k3*) to substantiate the *in vitro* findings whereby CO increased LPS-induced p38 activation. Another rationale for using *Mkk3*$^{-/-}$ mice was that CO selectively increased the LPS-induced p38 MAP kinase while affecting neither the ERK1/ERK2 nor JNK pathways. We gave a sublethal dose of LPS (1 mg/kg) to *Mkk3*$^{-/-}$ mice and *Mkk3*$^{+/+}$ littermates in the presence or absence of 250 p.p.m. CO. As expected, *Mkk3*$^{+/+}$ mice had a considerable increase in serum TNF-α production[23], whereas *Mkk3*$^{-/-}$ mice had lower serum TNF-α levels than did LPS-treated *Mkk3*$^{+/+}$ mice (Fig. 5*a*). This was expected, as LPS-induced production of TNF-α is regulated mainly, but not exclusively, through the MKK3–p38 MAPK pathway[14,21,24–26]. Furthermore, *Mkk3*$^{+/+}$ mice had less LPS-induced production of TNF-α in the presence of CO than did control mice treated with LPS alone. This inhibitory effect of CO was absent in *Mkk3*$^{-/-}$ mice: CO did not affect LPS-induced TNF-α production in *Mkk3*$^{-/-}$ mice. In addition, CO increased LPS-induced IL-10 levels in *Mkk3*$^{+/+}$ mice, compared with those in *Mkk3*$^{-/-}$ mice treated with LPS alone (Fig.

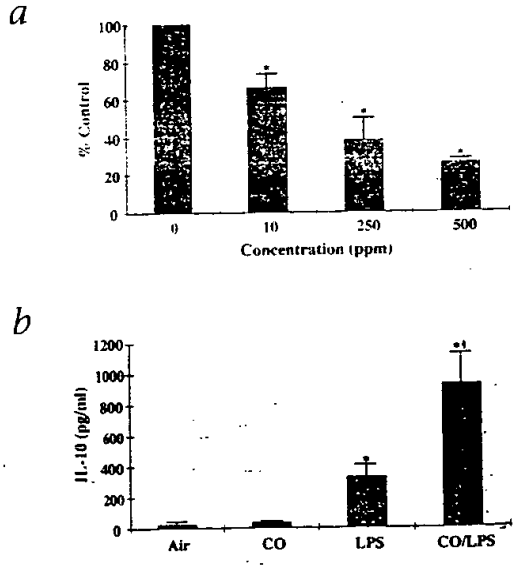

Fig. 3 CO inhibits LPS-induced production of TNF-α and IL-10. *a*, were exposed to CO (concentration, horizontal axis), and serum was measured by ELISA. *, $P < 0.0001$, compared with LPS alone. *b*, were exposed to CO, and serum IL-10 was determined by ELISA. 0.0001, compared with air or CO alone; ‡, $P < 0.002$, compared with Data represent the mean values ± s.e.m. from six to eight in mice in treatment group.

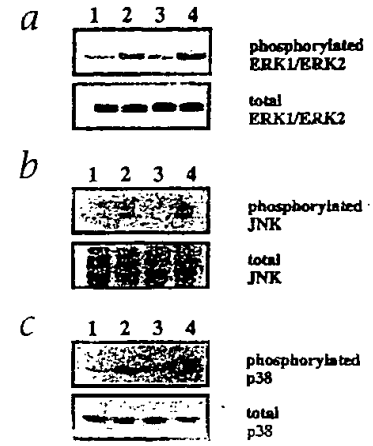

Fig. 4 Effects of CO on LPS-induced activation of MAP kinases. Western blot analysis of MAP kinase activation after 1 h of treatment with LPS in the absence or presence of CO: Lane 1, untreated control; lane 2, LPS; lane 3, CO alone; lane 4, LPS and CO. *a*, ERK1/ERK2. *b*, JNK. *c*, p38.

5*b*). However, *Mkk3*$^{-/-}$ mice did not have higher serum levels of IL-10 after administration of LPS in the presence of CO than did *Mkk3*$^{+/+}$ mice treated with LPS alone (Fig. 5*b*). Exposure to either air or 250 p.p.m. CO (data not shown) had no effect on serum production of TNF-α or IL-10 in either *Mkk3*$^{-/-}$ or *Mkk3*$^{+/+}$ mice (Fig. 5*a* and *b*).

Inhibition of TNF-α occurs by an IL-10-independent pathway

IL-10 is an inhibitor of pro-inflammatory cytokine synthesis and as such can limit the inflammatory process including TNF-α production[11]. Therefore, the increase in LPS-induced production of IL-10 in the mice treated with CO and LPS might have been responsible for the inhibition of TNF-α production. To test this, we administered LPS to IL-10-deficient mice in the presence or absence of 250 p.p.m. CO. Serum TNF-α production at 1 hour inhibited in both IL-10-deficient as well as wild-type control mice (60% and 73% inhibition, respectively), indicating that the decrease in TNF-α levels in those mice exposed to CO and LPS was not due to increased IL-10 production. *In vitro* studies using neutralizing antibodies to IL-10 in macrophages produced similar results, in that CO-mediated TNF-α suppression occurred independently of IL-10 (data not shown).

CO exerts post-transcriptional regulation of TNF-α production

We attempted to delineate the mechanism by which CO increases the p38 pathway while downregulating TNF-α production. RAW 264.7 cells showed increased TNF-α mRNA after LPS treatment in the presence of CO, similar to levels in cells treated with LPS alone (Fig. 6*a*). Cell lysates and media from these cells showed decreased TNF-α protein expression, by western blot analysis (Fig. 6*b* and *c*), after LPS when in the presence of CO. There was also decreased TNF-α production (more than 80% inhibition) in the media from these same cells, by ELISA analysis, confirming our previous results (Fig. 2*a*).

Discussion

There is strong evidence to support the emerging paradigm that HO-1 is essential in maintaining cellular and tissue homeostasis in various *in vitro* and *in vivo* models of oxidant-induced injury. Despite convincing data indicating the protective function of HO-1 in oxidative stress, the precise mechanism by which HO-1 serves as a potent cytoprotectant remains elusive. The catalytic by-products of heme catabolism, including bilirubin and ferritin (the latter generated by released iron), have been suggested to mediate the protective function of HO-1 (refs. 27–29). Both bilirubin and ferritin can function as potent anti-oxidant molecules *in vivo* and *in vitro* and as such are capable of eliciting cytoprotection[27–29]. CO, the remaining principal end-product of heme catalysis by HO, may be involved as a chemical messenger in neuronal transmission and modulation of vasomotor tone[18,19]. However, little is known regarding its functional involvement in potential cytoprotection against oxidative stress.

Our study shows an important physiologic function for CO in mammalian systems. We have demonstrated here that CO can exert potent anti-inflammatory effects in a concentration-dependent manner in both *in vivo* and *in vitro* models of LPS-induced inflammation (Fig. 2 and 3), similar to the results obtained with HO-1 (Fig. 1). The concentrations of CO (range, 10–500 p.p.m.) used here are comparable to the levels used in humans (0.03%) during measurement of D_LCO (lung diffusion capacity for carbon monoxide), a standard pulmonary function test, although our studies involved continuous CO exposure. Furthermore, extensive studies of long-term (2 years) exposure of rodents to low levels of CO (500 p.p.m.) reported no significant alterations in either physiological or biochemical parameters[30]. It is not apparent at present whether we are seeing physiological, pharmacological or toxic effects of CO. Careful and detailed studies will provide better insights into addressing this. A new aspect of our observation is that CO even at low concentrations can selectively modulate the pro-inflammatory/anti-inflammatory cascade of cytokines: CO selectively inhibited the LPS-induced production of TNF-α, IL-1β and MIP-1β while increasing IL-10 production in these same cells. Unexpectedly, these biological effects of CO did not involve the cGMP pathway (Table 1), which may be involved in the effects of

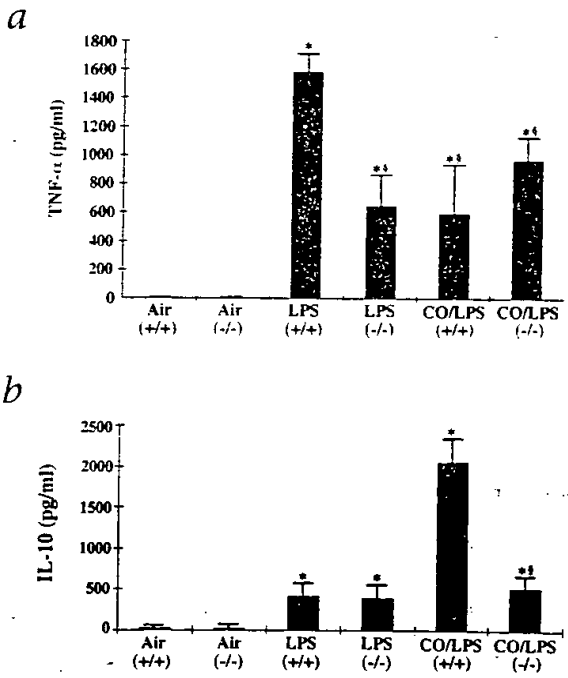

Fig. 5 Effects of CO on LPS-induced serum production of TNF-α and IL-10 in *Mkk3*$^{-/-}$ mice. *a*, *Mkk3*$^{+/+}$ or *Mkk3*$^{-/-}$ mice were exposed to CO, and serum TNF-α was determined by ELISA. *, $P < 0.0001$, compared with air alone; §, $P < 0.03$, compared with LPS$^{+/+}$. *b*, *Mkk3*$^{+/+}$ or *Mkk3*$^{-/-}$ mice were exposed to CO and serum IL-10 was determined by ELISA. *, $P < 0.001$ compared with air or CO alone; §, $P < 0.001$ compared with CO/LPS (+/+). Data represent the mean value ± s.e.m. of six to eight in mice in each treatment group.

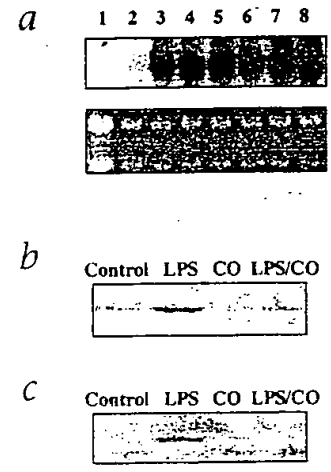

Fig. 6 Effect of CO on LPS-induced TNF-α expression. ***a***, Total RNA was isolated from RAW 264.7 cells after treatment with LPS in the absence or presence of CO, and was analyzed for TNF-α mRNA expression by northern blot analysis. Lane 1, air (control); lane 2, CO; lanes 3–5, LPS; lanes 6–8, LPS and CO. Below, ethidium bromide staining shows equal loading of samples. ***b*** and ***c***, Western blot analysis of TNF-α in cell lysates (*b*) or culture media (*c*) after treatment with LPS in the absence or presence of CO. Data represent four independent experiments.

CO seen in the neuronal and vascular systems, akin to NO (refs. 18,19). Furthermore, our data do not support the idea that CO may be mediating the anti-inflammatory effects through the production of NO, whose anti-inflammatory effects have been described[21]. Instead, these anti-inflammatory effects of CO seem to involve the MAP kinase signaling pathway, in particular the MKK3/p38 MAP kinase pathway (Figs. 4 and 5).

The essential involvement of the p38 MAP kinase pathway in mediating the functional anti-inflammatory actions of CO is based on several observations. CO selectively affected the LPS-induced activation of p38 MAP kinase while having no effect on either the ERK1/ERK2 or JNK MAP kinases (Fig. 4). Moreover, the attenuation of LPS-induced production of TNF-α by CO in *Mkk3*$^{+/+}$ mice was not seen in *Mkk3*$^{-/-}$ mice (Fig. 5*a*). Finally, the increase in LPS-induced IL-10 by CO in *Mkk3*$^{+/+}$ mice was not found in *Mkk3*$^{-/-}$ mice (Fig. 5*b*). This was consistent with findings showing that IL-10 production after treatment with LPS is regulated mainly by p38 (ref. 31). Indeed, the p38 MAP kinase pathway mediates HO-1 gene expression in response to oxidative stress in cultured hepatoma cells[32]. This essential function of MKK3/p38 MAP kinase in regulating endogenous induction of the protective HO-1 enzyme is consistent with our observations that this same pathway is essentially involved in mediating the biological effects of CO, a principal byproduct of heme catabolism by HO-1. Perhaps this represents a mechanism by which HO-1 and CO are amplified by p38 to exert functional anti-inflammatory effects.

The precise biochemical mechanism by which CO modulates the MAP kinases remains unclear at this time. Given that none of the upstream kinases in the MAP kinase pathway contains a heme moiety, a common target for CO, and our observation that CO mediated anti-inflammatory effects through cGMP–guanylyl-cyclase- and NO-independent pathways, CO could be modulating the upstream kinases through an unknown or unidentified intermediate molecule. This is supported by our observation that the protective effects of CO require new protein synthesis in a model of apoptosis induced by TNF-α (L.E.O. *et al.*, unpublished observations).

Our studies also indicate that CO inhibits LPS-induced production of TNF-α through an IL-10-independent pathway. Many reports have shown that reduced TNF-α production independent of increased IL-10 release can also occur in other systems including increases in cAMP, decreased intracellular calcium or β-adrenoceptor stimulation[33–35]. Our data have raised the question of how the increase in p38 MAP kinase activity, a potent intermediate signal transducer involved in the production of TNF-α after LPS administration[24,25], could be associated with decreased production of TNF-α, detected by ELISA and western blot analysis in the presence of CO. LPS-induced TNF-α gene expression was not affected by CO, as determined by northern blot analysis, whereas CO considerably inhibited LPS-induced TNF-α protein expression, as determined by western blot analysis (Fig. 6*b* and *c*) as well as by ELISA of culture media (Fig. 2*a*). It seems that CO is exerting post-transcriptional or secretional regulation of LPS-induced expression of TNF-α. Perhaps a delicate balance exists in the actions of p38, in that subtle cellular activation, as with LPS alone, is stimulatory for TNF-α synthesis, whereas hyperstimulation, as seen in cells treated with CO and LPS, becomes inhibitory and thus results in post-transcriptional downregulation of TNF-α production. This modulation by CO of LPS-induced production of TNF-α exemplifies accumulating evidence emphasizing the complexity of the molecular regulation of TNF-α expression[36,37]. Complex transcriptional and translational control of TNF-α expression has also been reported in other, similar systems[38–39].

The ability of CO to suppress pro-inflammatory responses in macrophage activation may also contribute to the suppression of xenograft rejection (Sato *et al.*, unpublished observations). Those studies mimicked the ability of HO-1 itself to protect against cardiac xenograft rejection shown previously[39]. In the latter study[39] beneficial effects of HO-1 were shown in avoiding rejection of a xenograft. CO probably mediates these protective effects in part by modulating platelet function, inducing vasodilatation and/or by inhibiting the pro-inflammatory response of monocytes, as described here. The anti-inflammatory effects of CO may also have been important in results showing both exogenous administration of HO-1 by gene transfer or CO inhalation protect against hyperoxia-induced lung injury and inflammation[7,40].

Our study shows a previously unknown physiological function for CO in a model of LPS-induced inflammation. Despite the longstanding accepted paradigm that CO as present in the environment is toxic or even lethal, our data indicate that CO at low concentrations can serve as a potent anti-inflammatory molecule. CO might be used therapeutically either by (over)expressing HO-1, by inducing HO-1 by genetic engineering or by local CO administration in areas of inflammation. CO, therefore, might be used therapeutically to treat septic shock syndrome and other inflammatory disease states.

Methods

Animals. Male C57BL/6 and IL-10-deficient mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice were allowed to acclimate for 1 week with rodent chow and water *ad libitum*. *Mkk3*$^{-/-}$ mice were generated as described[41]. Wild-type littermates were used as controls. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Animal Care and Use Committee of Yale University School of Medicine.

Reagents. All reagents were purchased from Sigma unless otherwise specified.

Plasmid construct. pSFFV/HO-1 was constructed by insertion of the HO-1 cDNA (ref. 49) downstream of the Friend spleen focus-forming virus 5′ long terminal repeat in the mammalian expression vector pSFFV/neo (ref. 43) (provided by S. Korsmeyer).

Transfection. Stably transfected cells were produced with the calcium phosphate precipitation technique as described[44]. RAW cells were plated at a density of 1×10^6/10 cm plate and, 16 h later, were transfected with 10 mg pSFFV/neo or pSFFV/HO-1. Cells were exposed to DNA–calcium

phate precipitate for 6 h, 'shocked' by treatment for 1 min with glycerol in phosphate-buffered saline, and cultured for an additional 24 h in complete medium before the addition of G418. Transfected cells were selected over a 2-week period in the presence of G418 (up to 800 mg/ml) and individual clones were isolated by limited dilution.

Cell culture experiments. RAW 264.7 mouse peritoneal macrophages were purchased from American Tissue Cell Culture (Rockville, Maryland) and primary cultures of rat pulmonary vascular smooth muscle cells were collected from male Sprague Dawley rats (250–300 g in body weight) as described[45]. Both cell types were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 µg/ml gentamicin in a humidified atmosphere of 5% CO_2 in air or 250 p.p.m. CO, 5% CO_2 in air. After 2 h of pretreatment with either CO or air, 1 µg/ml LPS or sterile saline was added to the culture media and the culture plates were returned to the chamber(s). Culture media and cells were collected at 1 h (TNF-α, IL-1β, MIP-1β and MIP-2) and 16 h (IL-10) for cytokine determination by ELISA and/or western and northern blot analyses.

Carbon monoxide exposures. Mice or macrophages were exposed to compressed air or varying concentrations of CO (0–500 p.p.m.). For cell culture experiments, 5% CO_2 was also present for buffering requirements. CO at a concentration of 1% (10,000 p.p.m.) in compressed air was mixed with compressed air with or without CO_2 in a stainless steel mixing cylinder before being delivered into the exposure chamber. Flow into the 3.70-ft^2 plexiglass animal chamber was maintained at rate of 12 l/min and into the 1.2-l cell culture chamber, at a rate of 2 l/min. The cell culture chamber was humidified and maintained at 37 °C. A CO analyzer (Interscan, Chatsworth, California) was used to measure CO levels continuously in the chambers. Gas samples were introduced to the analyzer through a port in the top of the chambers at a rate of 1 l/min and were analyzed by electrochemical detection, with a sensitivity of 10–600 p.p.m.. Concentration levels were measured hourly and there were no fluctuations in the CO concentrations after the chamber had equilibrated (approximately 5 min).

Animal experiments. Blood (0.25 ml) was obtained from the retroorbital sinus of mice, then the mice were exposed to 250 p.p.m. CO or room air for 1 h before intraperitoneal administration of 1 mg/kg LPS, *Escherichia coli* serotype 0127:B8. At 1 and 16 h thereafter, mice were removed from the exposure apparatus individually and a blood sample was obtained through the retroorbital sinus. After each blood collection, the mice were immediately returned to the exposure chamber. For hypoxia studies, mice were pre-treated for 1 h with 10% oxygen (hypoxia) and then given 1 mg/ml LPS intraperitoneally. At 1 h thereafter, they were removed from the exposure chamber individually and a blood sample was collected from the retroorbital sinus, and the serum was analyzed for TNF-α by ELISA. The oxygen concentration in the chamber was verified by an oxygen sensor calibrated for low oxygen tensions (VWR, Boston, Massachusetts).

Cytokine analysis. Serum and media samples were analyzed with ELISA kits purchased from R&D Systems (Minneapolis, Minnesota), using the manufacturer's instructions.

Western blot analysis. Assay kits purchased from New England Biolabs (Beverly, Massachusetts) were used following the manufacturer's instructions. At various times, cells were removed from the exposure chamber and rinsed with cold PBS and then 200 µl sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris HCl, pH 6.8, 2% weight/volume SDS, 10% glycerol, 50 mM DTT and 0.1% weight/volume bromphenol blue) was added to each plate. Cells were scraped from the plates and sonicated for 5 s. Of each sample, 20 µl was boiled for 5 min and then separated by 12% PAGE at 125 V for 90 min in Tris–glycine–SDS running buffer. The gel was transferred overnight at 40 V onto nitrocellulose membrane. Membranes were then incubated for 3 h with blocking buffer (5% nonfat dry milk in TTBS (10% Tween in Tris-buffered saline), washed with TTBS, and then incubated overnight in the appropriate rabbit polyclonal primary antibody against phosphorylated ERK, p38 or JNK. HO-1 and TNF-α blots were probed for 1 h in blocking buffer with rabbit antibody against HO-1 (Stressgen, Victoria Canada) or TNF-α (Santa Cruz Biotechnology, Santa Cruz, California), respectively. After incubation with primary antibody, the membranes were washed in TTBS and proteins were visualized using horseradish-peroxidase-conjugated antibody against rabbit IgG and the enhanced chemiluminesence assay (Amersham), according to the manufacturer's instructions. All MAP kinase blots were subsequently 'stripped' using standard stripping solution (100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.8) at 50 °C, and were re-probed with rabbit polyclonal antibody targeting total non-phosphorylated ERK, p38 or JNK, to confirm equal loading of samples.

RNA extraction and northern blot analysis. Total RNA was isolated using the Trizol method, with homogenization of the lung tissues in Trizol lysis buffer followed by chloroform extraction (Life Technologies).Total RNA (10 µg) was separated by 1% agarose gel electrophoresis and then transferred to GeneScreen Plus nylon membrane (NEN) by capillary action. Ethidium bromide staining of the gel was used to confirm the integrity and equal loading of the RNA. The nylon membranes were then prehybridized at 65 °C for 2 h in hybridization buffer (1% bovine serum albumin, 7% SDS, 0.5 M phosphate buffer, pH 7.0, and 1.0 mM ethylenediamine tetraacetic acid (EDTA)), followed by incubation at 65 °C for 24 h with hybridization buffer containing ^{32}P-labeled mouse TNF-α cDNA. The cDNA was labeled with ^{32}P-CTP using the random primer kit from Boehringer. Nylon membranes were then washed for 15 min twice each at 65 °C in wash buffer A (0.5% bovine serum albumin, 5% SDS, 40 mM phosphate buffer, pH 7.0, and 1 mM EDTA) followed by washes for 15 min three times each at 65 °C in buffer B (1% SDS, 40 mM phosphate buffer, pH 7.0, and 1.0 mM EDTA).

Nitrate/nitrite analysis. Both nitrate and nitrite were measured in cell culture media that had been ultrafiltered to remove all proteins with a molecular weight greater than 10 kDa. All samples were analyzed using assay kit protocols (R & D Systems, Minneapolis, Minnesota).

cGMP radioimmunoassay in macrophages. Macrophages or vascular smooth muscle cells were exposed to 250 p.p.m. CO or air. Then, 20 min before the end of the exposure, 1 mM IBMX (3-isobutyl-1-methylxanthine) was added to the cells to prevent phosphodiesterase degradation of cGMP. After 2 h of exposure, cells were removed from the exposure chamber and rinsed twice with PBS. Then, 0.5 ml of 75% EtOH was added and cells were scraped from the plate and disrupted through sonication for 5 s. Samples were then centrifuged at 2,000*g* for 10 min. Supernatants were transferred to fresh tubes and evaporated to dryness. The concentration of cGMP in the cell extracts was determined by radioimmunoassay (NEN) according to manufacturer's instructions. cGMP was normalized to protein concentration as determined by Bradford assay.

Statistical analysis. Data are expressed as the mean ± s.e.m. Differences in measured variables between experimental and control group were assessed using Student's *t*-tests. Statistical calculations used a Macintosh personal computer with the Statview II Statistical Package (Abacus Concepts, Berkeley, California). Results were considered statistically significant at $P < 0.05$.

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